Dimethylfumarate alleviates early brain injury and secondary cognitive deficits after experimental subarachnoid hemorrhage via activation of Keap1-Nrf2-ARE system

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OBJECTIVE Oxidative stress and the inflammatory response are thought to promote brain damage in the setting of subarachnoid hemorrhage (SAH). Previous reports have shown that dimethylfumarate (DMF) can activate the Kelch-like ECH-associated protein 1–nuclear factor erythroid 2-related factor 2–antioxidant-responsive element (Keap1-Nrf2-ARE) system in vivo and in vitro, which leads to the downregulation of oxidative stress and inflammation. The aim of this study was to evaluate the potential neuroprotective effect of DMF on SAH-induced brain injury in rats.

METHODS Rats were subjected to SAH by the injection of 300 μl of autologous blood into the chiasmatic cistern. Rats in a DMF-treated group were given 15 mg/kg DMF twice daily by oral gavage for 2 days after the onset of SAH. Cortical apoptosis, neural necrosis, brain edema, blood-brain barrier impairment, learning deficits, and changes in the Keap1-Nrf2-ARE pathway were assessed.

RESULTS Administration of DMF significantly ameliorated the early brain injury and learning deficits induced by SAH in this animal model. Treatment with DMF markedly upregulated the expressions of agents related to Keap1-Nrf2-ARE signaling after SAH. The inflammatory response and oxidative stress were downregulated by DMF therapy.

CONCLUSIONS DMF administration resulted in abatement of the development of early brain injury and cognitive dysfunction in this prechiasmatic cistern SAH model. This result was probably mediated by the effect of DMF on the Keap1-Nrf2-ARE system.

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KEY WORDS dimethylfumarate; subarachnoid hemorrhage; Nrf2; vascular disorders

SUBARACHNOID hemorrhage (SAH) is a critical neurosurgical phenomenon that is often coupled with several interrelated complications such as acute/delayed cerebral vasospasm, brain edema, obstructivecommunicating hydrocephalus, diffuse/focal cerebral ischemia or infarction, and lasting cognitive deficits. In China, the SAH-related morbidity rate is about 1.75 per 10,000. Despite recent progress in microsurgical and endovascular surgical techniques, the outcome of patients who suffer an SAH remains disappointing.

ABBREVIATIONS ARE = antioxidant-responsive element; DMF = dimethylfumarate; EBI = early brain injury; ELISA = enzyme-linked immunosorbent assay; EMSA = electrophoretic mobility shift assay; FJB = Fluoro-Jade B; GSH-Px = glutathione peroxidase; GST-α1 = glutathione S-transferase α1; HO-1 = heme oxygenase 1; IL = interleukin; Keap1 = Kelch-like ECH-associated protein 1; MDA = malondialdehyde; MWM = Morris water maze; NQO1 = quinone oxidoreductase 1; Nrf2 = nuclear factor erythroid 2-related factor 2; PBS = phosphate-buffered saline; SAH = subarachnoid hemorrhage; SOD = superoxide dismutase; TNFα = tumor necrosis factor-α.

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Our previous study demonstrated that the activation of nuclear factor erythroid 2-related factor 2 (Nrf2)–mediated antioxidative signaling may lead to diminution of the degree of early brain injury (EBI) after SAH. At the same time, antiinflammatory therapy has also been proven to have beneficial effects in SAH models. Dimethylfumarate (DMF), the main ingredient of an oral formulation of fumaric acid, was recently shown in pivotal Phase III trials to ameliorate the course of relapsing-remitting multiple sclerosis. Basic research has shown that the neuroprotective effects of DMF may be functionally attributable to its ability to upregulate the signaling system of the Kelch-like ECH-associated protein 1 (Keap1)-Nrf2–antioxidant response element (ARE) pathway and inhibit the expression of multiple neuroinflammatory mediators. We hypothesized that DMF would attenuate the development of EBI and promote neurobehavioral recovery after SAH, mechanisms that may involve Keap1-Nrf2-ARE–mediated antioxidative and antiinflammatory actions.

**Methods**

**Animals**

Male Sprague-Dawley rats (weighing 300–350 g) were purchased from the Animal Center of Soochow University (Suzhou, China). All procedures were approved by the Institutional Animal Care Committee and were in accordance with guidelines of the National Institutes of Health on the care and use of animals.

**Rat SAH Model and Physiological Variable Monitoring**

Each rat was anesthetized intraperitoneally with urethane (1000 mg/kg), and its head was fixed in a stereotactic frame on heating pads. Rectal temperature was recorded and maintained at 37°C throughout the surgery and the recovery period. Mean arterial blood pressure was monitored with polyethylene tubing (PE50) that was cannulated into the tail artery. At the same time, the arterial blood gas was measured by a blood gas analyzer (BJ05–840, Bayer) 3 times (before and after surgery and before planned death). The experimental SAH model was produced by the injection of 300 μl of autologous blood into the prechiasmatic cistern; the needle, with a rounded tip and a side hole, was inserted with stereotactic guidance, according to the procedure in our previous study.

**Experimental Design**

We established the following 4 experimental groups in a randomized fashion: 1) the control group (n = 20); 2) the SAH group (n = 20); 3) the SAH+vehicle group (n = 20); and 4) the SAH+DMF group (n = 20). Rats in the SAH+DMF group received 15 mg/kg DMF orally twice daily for 2 days after injury. Rats in the SAH+vehicle group received equal volumes of vehicle (saline) on the same schedule. In the first experimental setting, the animals were decapitated 48 hours after SAH, and tissue assays were performed (n = 10 in each group). In the second experiment, the animals were trained and evaluated in a Morris water maze (MWM) (n = 10 in each group).

**TUNEL and Fluoro-Jade B Staining**

Apoptosis was detected using TUNEL according to the manufacturer’s protocol (DeadEnd fluorometric kit; Promega). Fluoro-Jade B (FJB) (Histo-Chem, Inc.) was used as a marker of neuronal injury. Every third coronary sections for each sample. The number of positively stained cells in each section was counted in 10 microscope fields (at ×200 magnification) throughout the identical regions of the studied brain, and the mean percentage per visual field was calculated. The entire process was conducted by 2 technicians blinded to the group assignments.

**Behavior Testing**

Spatial learning and memory, including cued learning procedures, spatial acquisition tasks, reference memory tasks, and working memory tasks, were assessed by using the MWM according to the process used in a previous study.

**Western Blot Analysis**

Western blot analysis was performed according to our previous study. We used primary antibodies directed against Keap1, Nrf2, and heme oxygenase 1 (HO-1) (all diluted 1:200; from Santa Cruz Biotechnology) in phosphate-buffered saline (PBS) plus Tween 20 (PBST) at dilutions of 1:200, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (diluted 1:8,000 in PBST; Sigma-Aldrich, Inc.) was used as a loading control.

**Nuclear Protein Extract and Electrophoretic Mobility Shift Assay**

Nuclear protein was extracted and quantified as described. Electrophoretic mobility shift assays (EMSAs) were performed using a commercial kit (Gel Shift Assay System; Promega) following the methods used in our laboratory. The Nrf2 oligonucleotide probe (5′-AGTTGAGGGGGACTTTCCCAGGC-3′) was end labeled with [γ-32P]ATP (Free Biotech). EMSAs were performed according to the protocols in our previous study.

**Immunohistochemistry Study**

Immunohistochemistry of formalin-fixed paraffin-embedded sections was performed to determine the immunoreactivity of Keap1, Nrf2, and HO-1 according to the protocols in our previous study. Sections were incubated with primary antibodies (all diluted 1:200; from Santa Cruz Biotechnology) for 1 hour at room temperature, followed by a 15-minute wash in PBS. Sections were incubated with horseradish peroxidase–conjugated IgG (1:500 dilution; from Santa Cruz Biotechnology) for 60 minutes at room temperature. 3′-Diaminobenzidine was used as the chromogen, and counterstaining was performed with hematoxylin. Sections incubated in the absence of a primary antibody were used as negative controls.
Quantitative Real-Time Polymerase Chain Reaction

The mRNA levels of HO-1, reduced nicotinamide adenine dinucleotide (phosphate) [NAD(P)H]–quinone oxidoreductase 1 (NQO1), and glutathione S-transferase α1 (GST-α1) were determined by quantitative real-time polymerase chain reaction according to the procedures in our previous study.26

Enzyme Activity Assay

The protein levels of NQO1 and GST-α1 were analyzed according to the protocols in our previous study.26 All values are expressed as nanomoles per minute per milligram of protein and were analyzed by an experienced research technician, who was blinded to the experimental condition.

Measurement of Oxidative Stress

Malondialdehyde (MDA) levels were determined by using the method described in previous studies.16,19 Tissue superoxide dismutase (SOD) enzyme activities were determined with the RANSOD SOD assay kit (Randox). A tissue glutathione peroxidase (GSH-Px) assay kit (Northwest Life Science Specialties) was used to determine tissue GSH-Px activity, and this assay is an adaptation of a method used in previous studies.16,20

Enzyme-Linked Immunosorbent Assay

The levels of inflammatory mediators were quantified using specific enzyme-linked immunosorbent assay (ELISA) kits for rats according to the manufacturers’ instructions (TNF-α from Diaclone; interleukin 1β [IL-1β] and IL-6 from Biosource Europe SA) and our previous study.6 Values are expressed as nanograms/gram of protein.

Statistical Analysis

All data are presented as means ± SEM. SPSS 12.0 was used for statistical analysis of the data. All data were subjected to 1-way ANOVA. Differences between the experimental groups were determined by Fisher’s least significant difference posttest. Statistical significance was inferred at a p value of < 0.05.

Results

General Observations

No significant changes in body weight, mean arterial blood pressure, temperature, or injected arterial blood gas data were detected in any of the experimental groups (data not shown). The mortality rate of the rats in the control group was 0% (0 of 20 rats), and it was 20% (15 of 75 rats) in the SAH groups. As shown in Fig. 1B, the rats in the SAH groups exhibited blood clots over the basal surface of the brainstem and the circle of Willis.

Supplement of DMF Ameliorated EBI After Experimental SAH

A significant increase (p < 0.05) in water content was detected in the brain samples 48 hours after SAH compared with rats in the control group (Fig. 1D). The mean value of the brain water content in the cortex was decreased by DMF administration (p < 0.05) compared with that in the SAH+vehicle group. Few TUNEL- or FJB-positive apoptotic cells were found in the control rat brains (Fig. 1A and C). In the SAH and SAH+vehicle groups, the apoptotic and necrotic indices in the cortex were found to be significantly higher than those in the control animals (p < 0.01) (Fig. 1A and C). There was no statistically significant difference between the SAH and SAH+vehicle groups (p > 0.05). In the SAH+DMF group, the number of TUNEL-positive or FJB-positive cells in the studied cortex was significantly lower than that in the SAH+vehicle group (p < 0.01) (Fig. 1A and C).

Behavior Testing

A representative trial from each group is shown in Fig. 2. For all the behavioral measurements, swimming speed and thigmotaxis (percentage of time spent in the perimeter of the pool) were evaluated, and we found no significant differences among the 4 groups. Rats can typically find the visible platform, but in this study, they usually failed in the first trial, which happened equally across all groups. There were no differences in the escape latencies and swimming speeds in the cued learning procedure among the 4 groups (p > 0.05). Spatial learning was also the same for all 4 groups during the 2nd and 3rd days after blood injection. Spatial learning deficits appeared during the 4th and 5th days in the SAH groups and did not appear in the control groups. The DMF group exhibited significantly shorter escape latencies than did the vehicle group during the 4th and 5th days (Fig. 2A and B). Animals in all groups learned to find the platform to escape from the water within each testing day (Fig. 2A–D). However, rats in the SAH group were significantly impaired compared with the controls (the 4th and 5th days); this impairment was alleviated by DMF but not by the vehicle. Repeated-measures ANOVA indicated a significant difference in escape latencies (p < 0.01; Fig. 2A) and swimming distances (p < 0.01; Fig. 2C) between the SAH and control groups, and these measures were also improved by DMF but not by the vehicle. When the escape latencies and swimming distances from all of the testing days were separated into the 4 daily trials, the escape latencies of the SAH group were significantly longer than those of the control group. The DMF group exhibited significantly shorter swimming distances than did the vehicle group (p < 0.01; Fig. 2B). However, regarding the swimming distances alone, the rats in the DMF group exhibited significant improvement in distances over those in the vehicle group on the 5th day (p < 0.01; Fig. 2D).

Effect of DMF on Keap1, Nrf2, and HO-1 Protein Expression in the Brain After SAH

To determine the effects of DMF on the Keap1-Nrf2 ARE pathway in the cortex after SAH, Western blot analysis was performed to detect the changes of Keap1, Nrf2, and HO-1 activity, as described above. Figure 3 shows low levels of Keap1, Nrf2, and HO-1 in the control group. On Day 2 (48 hours) after SAH, the levels of Keap1, Nrf2, and HO-1 were significantly increased in the
SAH and SAH+vehicle groups (p < 0.05; Fig. 3B). There was no statistically significant difference between the SAH and SAH+vehicle groups (p > 0.05; Fig. 3B). After DMF administration, the activities of Keap1, Nrf2, and HO-1 were markedly increased in the SAH+DMF group (p < 0.05; Fig. 3B). EMSA autoradiography of the Nrf2 DNA-binding activities of brain samples is shown in Fig. 3D. Low Nrf2-binding activities (weak EMSA autoradiography) were found in the control group. Compared with the control group, Nrf2-binding activities in the injured brains were significantly increased (p < 0.05) in the SAH and vehicle-treated groups. In the SAH+DMF group, the Nrf2-binding activities were significantly upregulated (p < 0.05) in brain tissue surrounding the blood clot sites after SAH. Immunohistochemical studies showed that positive expressions of Keap1-, Nrf2-, and HO-1-immunostained cells appeared in the SAH+DMF group 48 hours after SAH (p < 0.05; Fig. 4C).

**Effects of DMF on mRNA Expressions of HO-1, NQO1, and GST-α1 in the Cortex After SAH**

The mRNA levels of 3 genes, HO-1, NQO1, and GST-α1, were detected by quantitative real-time polymerase chain reaction. The mRNAs of these proteins were expressed at low levels in the control group. The levels of HO-1, NQO1, and GST-α1 mRNA were significantly increased in the cortex in the SAH and SAH+vehicle groups compared with the control group (p < 0.05). mRNA expression was not significantly different in the SAH and the SAH+vehicle groups (p > 0.05). The mRNA expressions of HO-1, NQO1, and GST-α1 in the SAH+DMF group were significantly increased (p < 0.05; Fig. 4C).
**FIG. 2.** Upper: Representative images of MWM trials of the rats in the 4 groups. Lower: Spatial learning and memory in the MWM. Shown are escape latency and swimming distance over 16 trials (A and C) and averaged for each day (B and D) over Days 2–5. Figure is available in color online only.
were significantly upregulated compared with those of the SAH+vehicle group (Fig. 4B).

Impact of DMF on Expression of Antioxidant and Detoxifying Enzymes in the Brain

As shown in Fig. 4D, the activities of NQO1 and GST-α1 were low in the control group. Compared with those in the control group, cortical levels of these antioxidant and detoxifying enzymes were greatly increased after SAH. DMF administration after SAH could lead to significantly increased NQO1 and GST-α1 activities in rat brain.

Influence of DMF on Oxidative Stress in the Cortex After SAH

The tissue MDA levels and tissue SOD and GSH-Px enzyme activities are shown in Fig. 5A–D. SAH significantly increased the tissue MDA levels (p < 0.05) and significantly decreased the tissue SOD and GSH-Px enzyme activities (p < 0.05) when compared with controls. DMF treatment has shown protective effects via significantly decreasing (p < 0.05) the elevated MDA levels and also significantly increasing the reduced antioxidant enzyme activities (SOD, p < 0.01; GSH-Px, p < 0.05).

DMF Treatment Decreased Cortical Levels of Proinflammatory Cytokines After SAH

Concentrations of IL-1β, TNF-α, and IL-6 were low in the control group (Fig. 5D). Compared with the control group, cortical levels of the 3 inflammatory cytokines were greatly increased after SAH (p < 0.05). As shown in Fig. 5D, DMF administration after SAH can lead to significantly decreased IL-1β, TNF-α, and IL-6 concentrations (p < 0.05).

Discussion

Dimethylfumarate is the methyl ester of fumaric acid, which was initially recognized as a very effective hypoxic cell radiosensitizer. Later, DMF combined with 3 other fumaric acid esters was licensed in Germany as oral therapy for psoriasis (Fumaderm).9 Other diseases, such as necrobiosis lipoidica, granuloma annulare, and sarcoidosis were also found in case reports or small patient series to respond to treatment with DMF.28 Recently, Phase III clinical trials found that DMF (BG-12) successfully reduced the relapse rate and time to progression of disability in people with multiple sclerosis.8 DMF is thought to have immunomodulatory properties without causing significant immunosuppression.2 DMF was recommended for approval in the European Union by the European Medicines Agency on March 21, 2013, as a peroral treatment for multiple sclerosis under the name Tecfidera. On March 27, 2013, the FDA approved the use of Tecfidera capsules to treat adults with relapsing forms of multiple sclerosis.

In previous research regarding the DMF and Keap1-Nrf2-ARE pathways,1,3,15 Linker et al. investigated whether DMF exerted neuroprotective effects that depended on the function of the Keap1-Nrf2-ARE oxidative stress response pathway in a model of multiple sclerosis. The data indicated that DMF can activate the Keap1-Nrf2-ARE system in vitro and in vivo, which offered a novel cyto-
protective modality that further augmented the natural antioxidant responses in multiple sclerosis tissue and was not yet targeted by other multiple sclerosis therapies. In both models of cardiac ischemia-reperfusion injury and renal fibrosis, previous studies also showed that DMF upregulates the expression of the Keap1-Nrf2-ARE pathway and protects the heart and kidneys.\(^1,3\) The role of the Nrf2 pathway in EBI after SAH was investigated in our previous article, which proved that the Nrf2-ARE pathway was activated in the brain after SAH and played a beneficial role in EBI development, possibly through inhibiting cerebral oxidative stress by inducing antioxidant and detoxifying enzymes.\(^5\) In this study, we researched the neuroprotective contribution of DMF on EBI after SAH, and the results indicate that DMF can activate the Keap1-Nrf2-ARE pathway after SAH and inhibit oxidative stress and the levels of multiple inflammatory cytokines in this prechiasmatic blood-injection model.

Cerebral oxidative stress and inflammation play an important role in the entire process of EBI after SAH.\(^4\) Free radicals can damage neurons and other major cell types in the brain by enhancing lipid peroxidation, protein oxidation and degradation, and DNA damage, which results in endothelial injury and blood-brain barrier breakdown by initiating apoptotic cascades or necrotic processes.\(^4,24\) Substantial evidence indicates a critical role of proinflammatory cascades in the development and maintenance of EBI after SAH.\(^13,17\) The Keap1-Nrf2-ARE pathway has been shown to repress oxidative stress and inflammatory responses in the brain in different animal models, including SAH models.\(^12,14,23,31,32\) Mice with the knock-out Nrf2 gene were shown to have more nuclear factor κB activa-
The current data demonstrate neuroprotective effects of DMF on SAH that may be mediated by the activation of the Keap1-Nrf2-ARE system, which could cause inhibition of oxidative stress and inflammation in the brain after experimental SAH.

Conclusions

To the best of our knowledge, this study is the first to evaluate the effects of DMF on the EBI and secondary neurobehavioral dysfunction in this experimental SAH model, as well as the effects of DMF on the Keap1-Nrf2-ARE antioxidative and anti-inflammatory pathways after SAH. We found that SAH upregulates the protein expressions of the Keap1-Nrf2-ARE pathway–related mediators, upstream (Keap1 and Nrf2) and downstream (HO-1, NQO1, and GST-α1) factors, in the brain tissue surrounding blood clots, which could be markedly upregulated by DMF therapy. These results suggest that SAH activates the Keap1-Nrf2-ARE signaling pathway in the rat brain, which might play a central role in the antioxidative and anti-inflammatory effects that lead to improved outcome after SAH. The therapeutic benefits of post-SAH DMF administration might be a result of its salutary effect on modulating the Keap1-Nrf2-ARE signaling pathway.

References


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